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Yeast Mitochondrial Division and Distribution Require the Cortical Num1 Protein

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SUMMARY

Yeast mitochondrial division requires the dynamin-related Dnm1 protein. By isolating high-copy suppressors of a dominant-negative Dnm1p mutant, we uncovered an unexpected role in mitochondrial division and inheritance for Num1p, a protein previously shown to facilitate nuclear migration. *num1* mutants contain an interconnected network of mitochondrial tubules, remarkably similar to cells lacking Dnm1p, and time-lapse microscopy confirms that mitochondrial fission is greatly reduced in *num1Δ* cells. We also find that Num1p assembles into punctate structures, which often colocalize with mitochondrial-bound Dnm1p particles. Suggesting a role for both Num1p and Dnm1p in mitochondrial inheritance, we find that *num1 dnm1* double mutants accumulate mitochondria in daughter buds and that mother cells are frequently devoid of all mitochondria. Thus, our studies have revealed an additional role for Dnm1p in mitochondrial transmission through its interaction with Num1p, thereby providing a link between mitochondrial division and inheritance.

INTRODUCTION

Mitochondria are dynamic organelles displaying a stunning array of shapes and numbers in different eukaryotic cell types (Bereiter-Hahn and Voth, 1994; Tandler and Hoppel, 1972). The shape and number of mitochondria are controlled, at least in part, by organelle fusion and division. In the yeast *Saccharomyces cerevisiae*, mitochondria in wild-type cells appear as several elongated, tubular-shaped organelles (Jensen et al., 2000; Okamoto and Shaw, 2005; Shaw and Nunnari, 2002). Mitochondrial fusion depends on the function of several different proteins, including the Fzo1p GTPase (Hermann et al., 1998; Rapaport et al., 1998). In *fzo1*Δ mutants, fission in the absence of fusion produces numerous fragmented organelles that rapidly lose mitochondrial DNA (mtDNA). In contrast, cells defective in division contain a single mitochondrion consisting of an interconnected network of tubules. Strikingly, normal mitochondrial tubules are seen in cells defective in both division and fusion, suggesting that a balance of the two activities is critical for normal shape and number of mitochondria (Sesaki and Jensen, 1999).

In yeast, the mitochondrial division machinery is composed of at least four proteins: Fis1p (Mozdy et al., 2000), Mdv1p (Tieu and Nunnari, 2000) (also called Gag3p [Fekkes et al., 2000] and Net2p [Cerveny et al., 2001; Cerveny and Jensen, 2003]), Caf4p (Griffin et al., 2005), and the dynamin-related GTPase Dnm1p (Bleazard et al., 1999; Otsuga et al., 1998; Sesaki and Jensen, 1999). Mdv1p and Caf4p associate with the mitochondrial surface through their interactions with the outer membrane protein Fis1p and are capable of forming homo- and hetero-oligomers with each other (Cerveny and Jensen, 2003; Griffin et al., 2005; Tieu et al., 2002). Both the Caf4 and Mdv1 proteins associate with Dnm1p via their carboxy-terminal WD-repeat domains. In *caf4 mdv1* double mutant cells, much of Dnm1p is dissociated from mitochondria, illustrating the partially overlapping functions of these homologous proteins (Griffin et al., 2005). Interestingly, *caf4* mutants do not exhibit dramatic defects in mitochondrial scission, suggesting that Mdv1p plays the major role in mitochondrial division.

Like all dynamin-related proteins, Dnm1p contains an amino-terminal GTPase domain, a highly conserved middle region, and a carboxy-terminal coiled-coil domain (Figure 1A) (Praefcke and McMahon, 2004). In wild-type yeast cells, Dnm1p-GFP can be found in about a dozen dot-like structures that dynamically associate with the mitochondrial surface, and some of these puncta appear to mark sites of mitochondrial division (Bleazard et al., 1999; Legesse-Miller et al., 2003; Otsuga et al., 1998; Sesaki and Jensen, 1999). In addition, a significant fraction of Dnm1p-GFP is localized to smaller moving structures in the cytosol (Jensen et al., 2000). Mutations that block Dnm1p's GTPase activity change the localization of Dnm1p from many puncta that come on and off mitochondria to several large complexes that more stably bind to the outer membrane, indicating that the GTPase cycle regulates Dnm1p's transient interactions with the organelle (Cerveny and Jensen, 2003). In contrast, Dnm1-109p, a dominant-negative form of Dnm1p carrying an amino acid change in the middle region (G350D), is diffusely

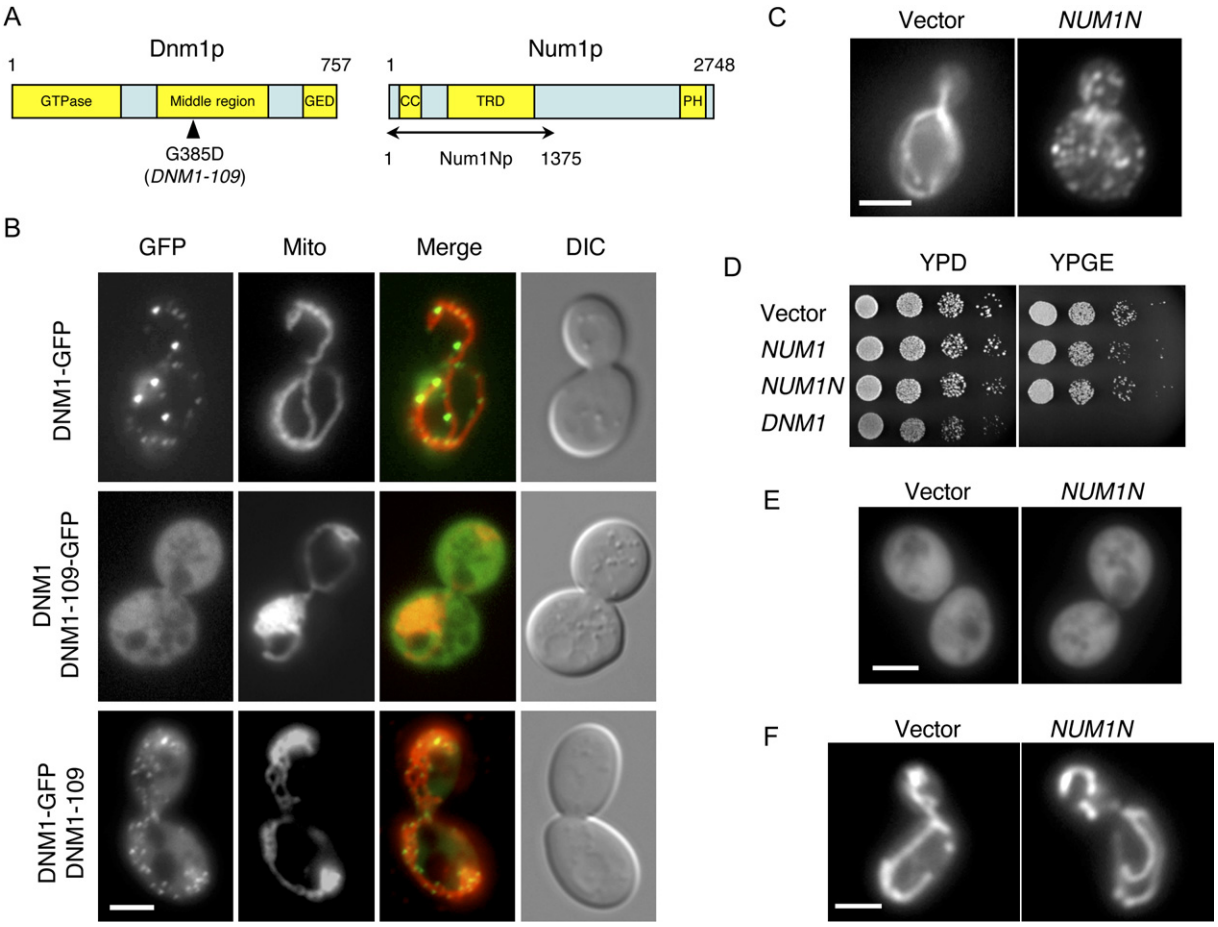


Figure 1. *NUM1* is a High-Copy Suppressor of the Dominant *DNM1-109* Mutant

(A) Domain structures of Dnm1p and Num1p; the *DNM1-109* mutation (G385D) is indicated by an arrowhead, and the Num1Np fragment is shown by a double-headed arrow.

(B) Dnm1-109p does not block assembly of wild-type Dnm1p into mitochondrial-associated particles. *dnm1*Δ strain YHS19 with pHS20 (Dnm1p-GFP; top panel of images), wild-type strain BY4741 with pHS21 (Dnm1-109-GFP; center panel), and *dnm1*Δ strain YHS19 with pHS27 (Dnm1-109p-HA) and pKC11 (Dnm1p-GFP; bottom panel) were stained with mitofluor red 589 (Mito) and examined by DIC and fluorescence microscopy. The scale bar is 3 μm.

(C) The expression of Num1Np suppresses *DNM1-109* by stimulating mitochondrial division. *fzo1*Δ *DNM1-109 DNM1* cells (YKC3B) were transformed with either 2μ-plasmid pKC1000 (*NUM1N*) or empty vector pRS426 and were then strained with mitofluor red 589 and examined.

(D) Num1Np does not cause loss of mtDNA in the absence of Dnm1p. *fzo1Δ dnm1Δ* cells (YHS27) were transformed with pRS426 (vector), pKC1001 (*NUM1*), pKC1000 (*NUM1N*), or pKC1005 (*DNM1*). 10-fold serial dilutions of cells were spotted on glucose-containing YPD plates and glycerol/ethanol-containing YPGE plates and were then incubated at 30°C for 2 (YPD) and 4 (YPGE) days. Lack of growth on YEPGE indicates loss of mtDNA.

(E) Num1Np does not recruit Dnm1-109p to mitochondria. *fzo1Δ DNM1* strain RJ2143 with pHS21 (Dnm1-109p-GFP) was transformed with either pRS426 (vector) or pRKC1000 (*NUM1N*) and examined.

(F) Overexpression of *NUM1N* does not cause fragmentation of mitochondria. Wild-type strain BY4741 expressing Cox4p-GFP from pHS12 was transformed with either pRS426 (vector) or pKC1000 (*NUM1N*) and was examined by fluorescence microscopy.

distributed throughout the cytosol, suggesting that the middle region is important for self-assembly and efficient binding to mitochondria (Jensen et al., 2000). Further supporting this idea, recent studies provide evidence that Dnm1-109p is defective in self-assembly (Bhar et al., 2006; Ingerman et al., 2005). To identify novel proteins that regulate the association of Dnm1p with mitochondria, we carried out a genetic screen to isolate multicopy suppressors of *DNM1-109*. Using this screen, we discovered an unexpected role for the cortical Num1 protein in mitochondrial division and distribution.

RESULTS

DNM1-109 Encodes a Dominant Inhibitor of Mitochondrial Division

A previous screen for mitochondrial morphology mutants identified 14 alleles of *DNM1*, 5 of which are dominant (Jensen et al., 2000; Sesaki and Jensen, 1999). Dnm1p-GFP localization studies showed that while many of the altered proteins accumulated on the mitochondrial surface in large aggregates, the protein encoded by *DNM1-109* was diffusely distributed throughout the cytosol

(Figure 1B). Interestingly, expression of the Dnm1-109 protein does not appear to prevent wild-type Dnm1p-GFP from associating with the mitochondrial surface in dot-like structures (Figure 1B). These data raise the possibility that Dnm1-109p blocks mitochondrial division by sequestering a cytosolic fission protein and preventing it from assembling with mitochondrial-bound Dnm1p.

Multiple Copies of *NUM1* Suppress the Dominant *DNM1-109* Mutant

To identify new Dnm1p-intereacting proteins, we isolated high-copy suppressors of the *DNM1-109* mutant. The basis for our screen is that *fzo1* cells rapidly lose their mtDNA, but this loss can be blocked by inactivation of Dnm1p function (Bleazard et al., 1999; Sesaki and Jensen, 1999). We constructed strain YKC3B, which contains *fzo1Δ*, *DNM1*, and the dominant-negative *DNM1-109* mutation, and found that these cells maintain their mitochondrial genome. To identify suppressors, we transformed YKC3B with a yeast genomic library carried on multicopy 2μ-plasmids and screened for transformants that now lose their mtDNA (see Experimental Procedures). While many of the plasmids we isolated carried genes encoding known division components, such as *DNM1* and *MDV1*, we also isolated a plasmid that encodes a truncated form of the Num1 protein. Num1p is a 313 kDa protein that contains an amino-terminal coiled-coil region, an internal tetratricopeptide repeat domain (TRD), and a carboxy-terminal pleckstrin homology (PH) domain (Figure 1A) (Kormanec et al., 1991). The *NUM1*-containing plasmid that suppressed *DNM1-109* contains only about half of the open reading frame (residues 1–1375), which we call Num1Np (Figure 1A).

Several observations suggest that Num1Np binds Dnm1-109p in the cytosol and thereby activates mitochondrial division. For example, overproduction of Num1Np leads to fragmentation of mitochondria in YKC3B (Figure 1C) and does not simply cause loss of mtDNA in *fzo1 dnm1* cells (Figure 1D). We also find that Num1Np does not recruit Dnm1-109p to the mitochondrial surface, but most likely sequesters it in the cytosol (Figure 1E). Since overproduction of *NUM1N* in wild-type cells does not cause fragmentation of mitochondria, we also argue that Num1Np does not act as an inhibitor of mitochondrial fusion (Figure 1F). We subsequently found that 2μ-plasmids expressing full-length Num1p also suppress *DNM1-109*, but not as efficiently as the Num1Np-expressing plasmid (data not shown).

num1Δ Cells Exhibit Aberrant Mitochondrial Structures, Similar to *dnm1Δ* Cells

Although a number of studies show that Num1p functions in nuclear migration (Bloom, 2001), we found that *num1*Δ mutants were also defective in mitochondrial shape and distribution. In contrast to the 5–10 discrete mitochondrial tubules in wild-type cells, fluorescence microscopy showed that *num1Δ* cells contained an interconnected network of tightly packed tubules, remarkably similar to those in *dnm1Δ* cells (Figure 2A). *num1Δ* cells showed more mitochondrial variation than *dnm1Δ* mutants, with shapes ranging from networks to more disorganized net-like structures and clustered/aggregated organelles (Figure 2B). Supporting our observations, we note that *num1*Δ was also uncovered in a genome-wide screen for yeast mitochondrial morphology mutants (Dimmer et al., 2002). In contrast to mitochondria, the structure of other organelles, such as the nucleus, ER, and vacuoles, was not disturbed in *num1*Δ cells, nor was the distribution of the actin cytoskeleton (Figure S1; see the Supplemental Data available with this article online).

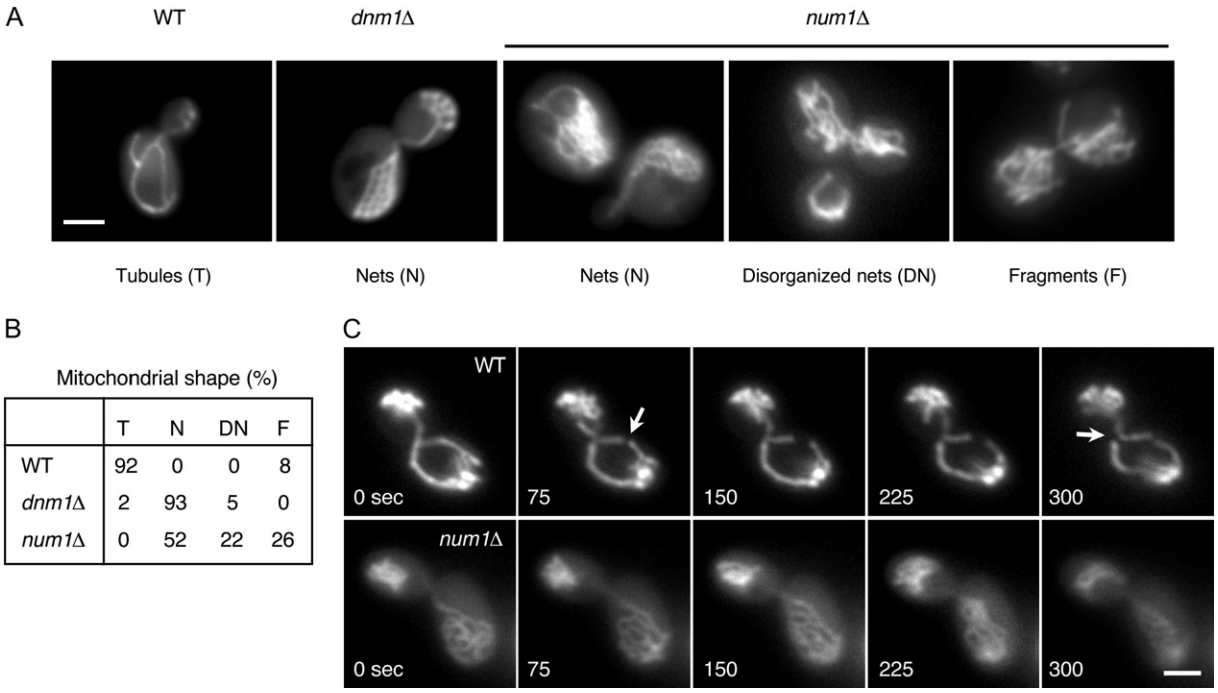
Num1p Is Critical, but Not Completely Essential, for Mitochondrial Division

The net-like mitochondria in *num1Δ* cells suggest that Num1p is involved in mitochondrial division. To test this possibility, we examined mitochondria by using time-lapse microscopy (Figure 2C). In wild-type cells, fission of mitochondria was seen approximately every 2–3 min. In contrast, in *num1Δ* cells, we did not see a single division event during our 5 min of observation, suggesting that mitochondrial division is strongly inhibited in *num1Δ* cells (n = 10). Nonetheless, several observations suggest that, unlike *dnm1*Δ mutants, at least some mitochondrial scission still occurs in *num1*Δ cells. For example, consistent with our previous studies (Sesaki and Jensen, 1999), *dnm1Δ fzo1Δ* cells contain tubular-shaped mitochondria, much like those seen in wild-type cells (Figure 3A). However, mitochondria in *num1Δ fzo1Δ* cells appear as multiple clusters of fragmented organelles, similar to mitochondria in the *fzo1Δ* mutant (Figure 3A). Also, while disruption of *DNM1* suppresses the mtDNA loss of *fzo1*Δ cells, *num1Δ fzo1Δ* cells were unable to grow on nonfermentable carbon sources, indicating that they lost their mtDNA (Figure 3B).

Also, suggesting that some organelle fission still occurs in cells lacking Num1p, we found that disruption of the actin cytoskeleton by Latrunculin A (Lat A) causes mitochondrial fragmentation in the *num1*Δ mutant (Figure 3C). Previous studies have shown that yeast mitochondria interact with actin (Boldogh et al., 1998; Drubin et al., 1993) and that LatA treatment of cells induces a Dnm1p-dependent division of the mitochondria (Jensen et al., 2000; Cerveny et al., 2001). When we treated wild-type and *num1Δ* cells with Lat A, we found that mitochondrial division in both wild-type and *num1Δ* cells produced partially fragmented organelles (Figure 3C). Lat A-induced division did not occur in *dnm1Δ* cells, and a single net-like organelle similar to that in the untreated *dnm1*Δ mutant was seen. We conclude that, unlike cells lacking Dnm1p, *num1*Δ mutants retain some fission ability. Perhaps yeast cells contain an activity that overlaps with Num1p function, much like the parallel roles played by the fission proteins Mdv1p and Caf4p (Griffin et al., 2005).

Num1p and Dnm1p Specifically Interact

Dnm1p is known to form dot-like structures that bind and release the mitochondrial surface (Cerveny and Jensen, 2003; Jensen et al., 2000; Legesse-Miller et al., 2003;



	T	N	DN	F
WT	92	0	0	8
dnm1Δ	2	93	5	0
num1Δ	0	52	22	26

Figure 2. Num1p Is Required for Normal Mitochondrial Shape and Division

(A) *num1*Δ and *dnm1Δ* cells contain similarly misshapen mitochondria. Wild-type (BY4741), *dnm1Δ* (YHS19), and *num1Δ* (RJ2142) cells expressing mitochondrial-targeted Cox4p-GFP from pHS12 were examined by fluorescence microscopy. The scale bar is 3 μm.

(B) Quantitation of mitochondrial morphology in the cells depicted in (A). The number of cells (out of 100 total) exhibiting mitochondrial tubules (T), nets (N), disorganized nets (DT), or fragments (F) is shown.

(C) *num1Δ* cells are defective in mitochondrial division. Wild-type (BY4741) and *num1Δ* (RJ2142) cells expressing Cox4p-GFP from pHS12 were examined by fluorescence time-lapse microscopy. Arrows indicate division events.

Naylor et al., 2006), while Num1p is reported to form puncta near the cell cortex (Farkasovsky and Kuntzel, 2001; Heil-Chapdelaine et al., 2000). When we examined the merged images of over 100 cells expressing functional Num1-RFP and Dnm1-GFP fusion proteins, we found that nearly 70% of the Num1-RFP dots colocalized with Dnm1-GFP (Figures 4A and 4B). Using confocal microscopy, we found that the Num1p and Dnm1p particles were usually adjacent and were organized in a side-by-side manner (Figure 4C).

In contrast to Dnm1p, we found that Mdv1p, another division component found in mitochondrial-associated particles (Cerveny et al., 2001; Tieu and Nunnari, 2000), showed no association with Num1p (Figures 4A and 4B). In our images, each of the GFP-Mdv1p and Num1p-RFP particles appeared distinct. Similarly, another outer membrane (OM) protein, Mmm1p, which forms dot-like structures (Aiken Hobbs et al., 2001), did not colocalize with Num1p (Figures 4A and 4B). Our data therefore suggest that there are at least two populations of Dnm1p: one associated with Num1p, and the other with Mdv1p (Cerveny et al., 2001; Tieu and Nunnari, 2000). Alternatively, Num1p and Mdv1p might consecutively interact with Dnm1p. It has recently been shown that Mdv1p is recruited to preassembled Dnm1p particles during mitochondrial division (Naylor et al., 2006). In this scenario, Num1p could dissociate from Dnm1p to unmask an Mdv1p-binding site.

Biochemical studies also indicate that Num1p interacts with Dnm1p. We purified Num1p from yeast cells expressing a Num1p-TAP fusion protein (Rigaut et al., 1999) by using IgG affinity chromatography. Western blots of the eluate fraction showed that a significant amount of Dnm1p (10%–30% of the total) copurified with Num1p-TAP (Figure 4D). In contrast, the fission protein, Mdv1p; the fusion component, Fzo1p; and the abundant outer membrane protein, OM45, did not bind to Num1p-TAP (Figure 4D). Cytosolic hexokinase and the β subunit of the mitochondrial ATPase showed no interaction with Num1p-TAP, and no Dnm1p was purified from cells lacking the TAP tag (data not shown). Thus, the interaction between Num1p and Dnm1p appears specific. We note that in different experiments the amount of Dnm1p that copurified with Num1p-TAP was appreciably variable, indicative of a weak and/or dynamic interaction between Dnm1p and Num1p.

Num1p Associates with Mitochondria Independent of Dnm1p and Fis1p

A recent proteomic study found that at least a fraction of Num1p is mitochondrial (Sickmann et al., 2003). Supporting this localization, we find, by using confocal microscopy, that most of the Num1p-GFP particles appear bound to the mitochondrial surface, often at the tips of the tubules (Figures 5Aa, 5Ad, and 5Ae). Supporting

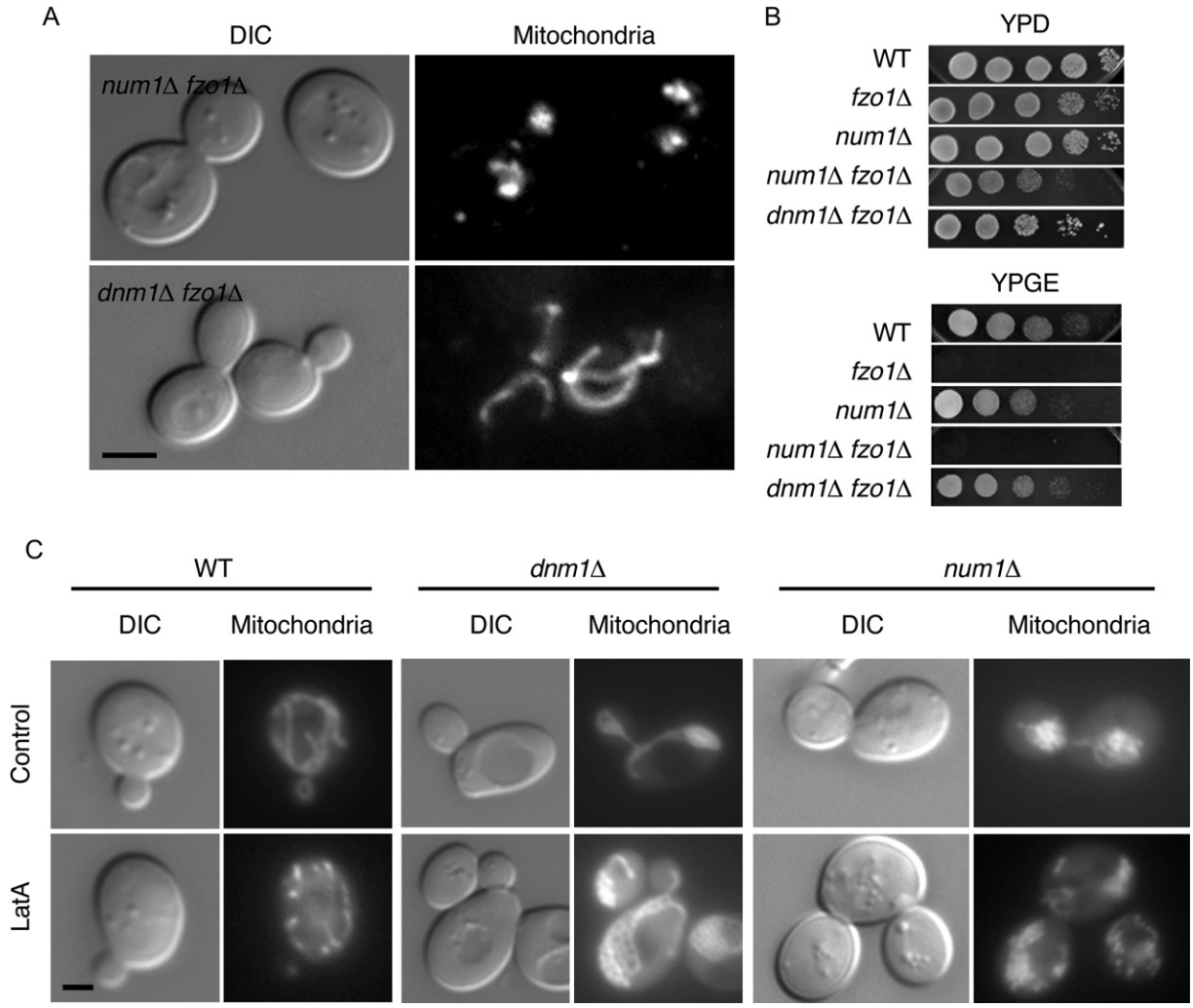


Figure 3. *num1Δ* Cells Retain Some Fission Ability

(A) Loss of Num1p does not rescue the mitochondrial shape defect in *fzo1*Δ cells. *dnm1*Δ *fzo1*Δ (YHS27) and *num1*Δ *fzo1*Δ (RJ2144) cells were grown in YEPGal at 30° to mid-log phase, stained with mitofluor red 589 to visualize mitochondria, and then examined.

(B) Deletion of *NUM1* does not rescue the growth defect of *fzo1Δ* cells. Wild-type (RJ2145), *num1Δ* (RJ2142), *fzo1Δ* (RJ2143), *num1Δ fzo1Δ* (RJ2144), and *dnm1Δ fzo1Δ* (YHS27) cells were grown in liquid YEPD, then diluted, spotted, and grown as in Figure 1D.

(C) In contrast to *dnm1*Δ cells, Latrunculin A treatment causes mitochondrial fragmentation in *num1Δ* cells. Wild-type (RJ2137), *dnm1Δ* (RJ2135), and *num1Δ* (RJ2134) cells expressing matrix-targeted Cox4-GFP from pHS12 were treated with either DMSO (Control) or Latrunculin A (Lat A) for 45 min at 24°C. The scale bar is 3 μm.

previous observations (Heil-Chapdelaine et al., 2000), Num1p-GFP also showed a crescent-like distribution along the cell cortex, which we also found to align with mitochondria (Figures 5Ac and 5Ad). Interestingly, the Num1p-mitochondrial interaction does not require Dnm1p. In *dnm1Δ* cells expressing Num1p-GFP, we found that Num1p still localized to puncta near the cell cortex, and that nearly all of the Num1-GFP dots were associated with the net-like mitochondria in the *dnm1*Δ mutant (Figures 5B and 5C). Strikingly, we often found a large particle of Num1p-GFP bound to the tip of the bundle. The crescent-like pattern of other, smaller Num1p-GFP structures cradled the mitochondrial nets. As shown in Figures 5B and 5C, we also observed that the mitochondrial association of Num1p is independent of Fis1p, Fzo1p, or Mmm1p. Although cortical localization of Num1p changes during different cell cycle stages (Heil-Chapdelaine et al., 2000), we found that similar amounts of Num1p were associated with mitochondria throughout the cell cycle (Figure S2).

Num1p Provides a Dnm1p-Docking Site on Mitochondria

Previous studies have shown that Dnm1p binds to the mitochondrial integral OM protein Fis1p, via the linker proteins Mdv1p and Caf4p (Cerveny and Jensen, 2003; Cerveny et al., 2001; Griffin et al., 2005; Karren et al., 2005; Mozdy et al., 2000; Tieu et al., 2002). We find that

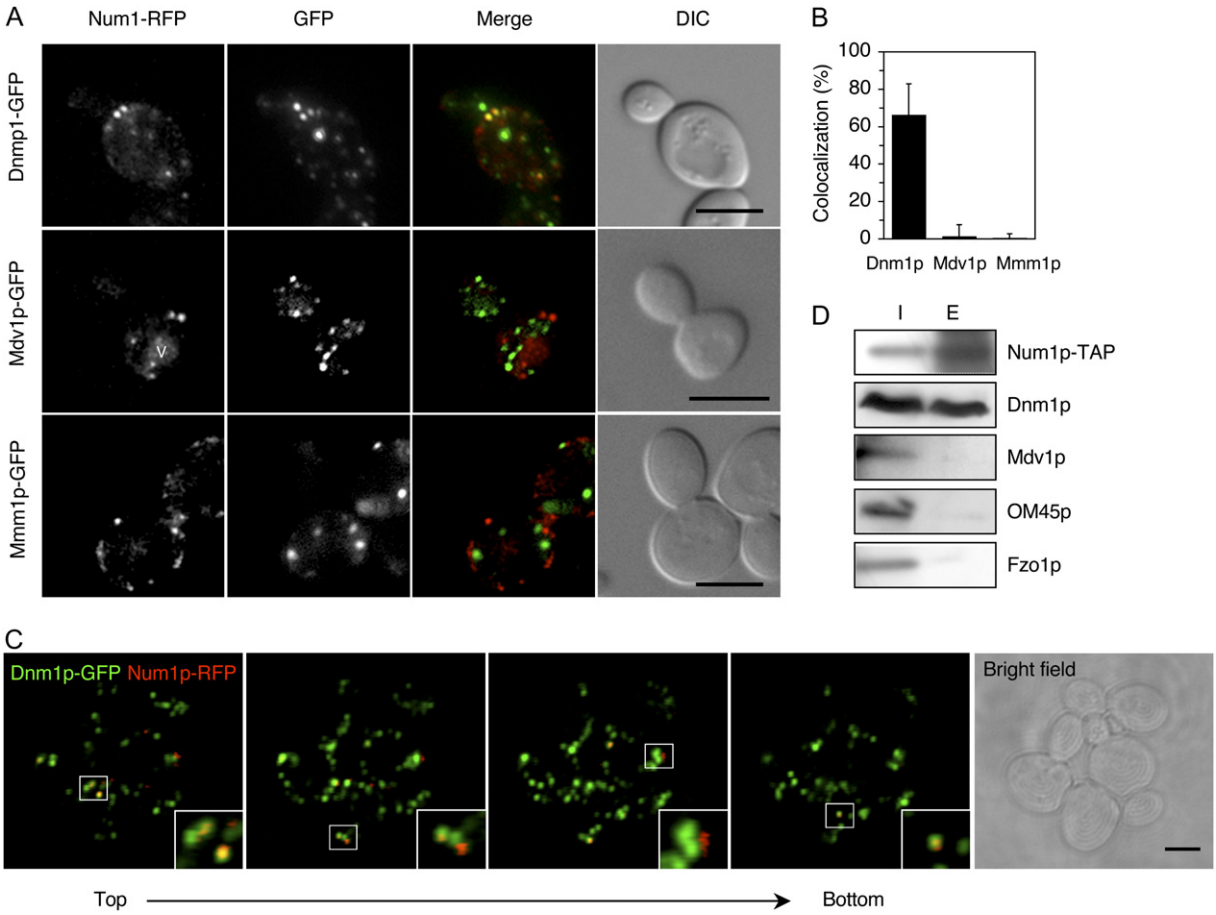


Figure 4. Num1p Physically Interacts with Dnm1p and Is Associated with Mitochondria

(A) Num1p colocalizes with Dnm1p, but not with Mdv1p or Mmm1p. *dnm1Δ NUM1-RFP* strain RJ2194 with pHS20 (Dnm1p-GFP) and *mmm1Δ NUM1-RFP* strain RJ2193 with pAA2 (Mmm1p-GFP) (Aiken Hobbs et al., 2001) were examined by wide-field fluorescence microscopy. *mdv1Δ NUM1-RFP* strain RJ2289 carrying pKC62 (pGAL-GFP-Mdv1p) (Cerveny and Jensen, 2003) was pregrown in SRaf, and the expression of GFP-Mdv1p was induced by the addition of 2% galactose for 30 min. The diffuse vacuolar (v) staining of Num1p-RFP seen in some cells is indicated. The scale bars are 3 μm.

(B) Quantitation of Num1p-RFP particles that colocalize with Dnm1p-GFP, GFP-Mdv1p, or Mmm1p-GFP. At least 27 cells of each type were examined. Error bars indicate SD.

(C) Confocal images, at 0.6 μm intervals, of *NUM1-RFP* strain RJ2194 with pHS20 (Dnm1p-GF) are shown from the top to bottom surface. The boxed regions in the merged images are magnified in the lower right corner.

(D) Dnm1p copurifies with Num1p. The Num1p-TAP fusion protein was purified with IgG-Sepharose resin. A total of 10% of the input (I) and 33% of the eluate (E) fractions were analyzed by SDS-PAGE, followed by western blotting with antibodies to the TAP tag of Num1p-TAP, Dnm1p, Mdv1p, OM45p, and Fzo1p.

Num1p may also provide an attachment site for Dnm1p. In particular, we found that some, but not all, Dnm1p fails to localize to the mitochondria in cells lacking either Num1p or Fis1p. In *num1*Δ cells and *fis1Δ* cells, we found that Dnm1p-GFP still assembled into dot-like structures (Figure 6A), but the amount of cytoplasmic Dnm1p-GFP increased nearly 3-fold compared to wild-type cells (Figure 6B). Furthermore, a significant increase in cytoplasmic Dnm1p-GFP particles was found in cells lacking both Num1p and Fis1p compared to either *num1Δ* or *fis1Δ* single mutants (Figures 6A and 6B). These results clearly demonstrate that Num1p mediates Fis1p-independent recruitment of Dnm1p to mitochondria. Further supporting this idea, we found that GFP-Mdv1p is normally distributed on mitochondria in the absence of Num1p (Figure S3).

Num1p Regulates Movements of Dnm1p Particles

Dnm1p is very dynamic in yeast cells, which can be seen in the rapid movements of Dnm1p-GFP particles (Jensen et al., 2000; Legesse-Miller et al., 2003). To probe the role of Num1p in Dnm1p dynamics, we examined the motility of Dnm1p-GFP in wild-type and *num1*Δ cells. We noticed that Dnm1-GFP-containing spots moved more rapidly, and with less directionality, in *num1Δ* cells compared to wild-type cells. In these studies, time-lapse images of Dnm1p-GFP in either wild-type cells (Figure 6C; see Movie S1) or the *num1Δ* mutant (Figure 6C; see Movie

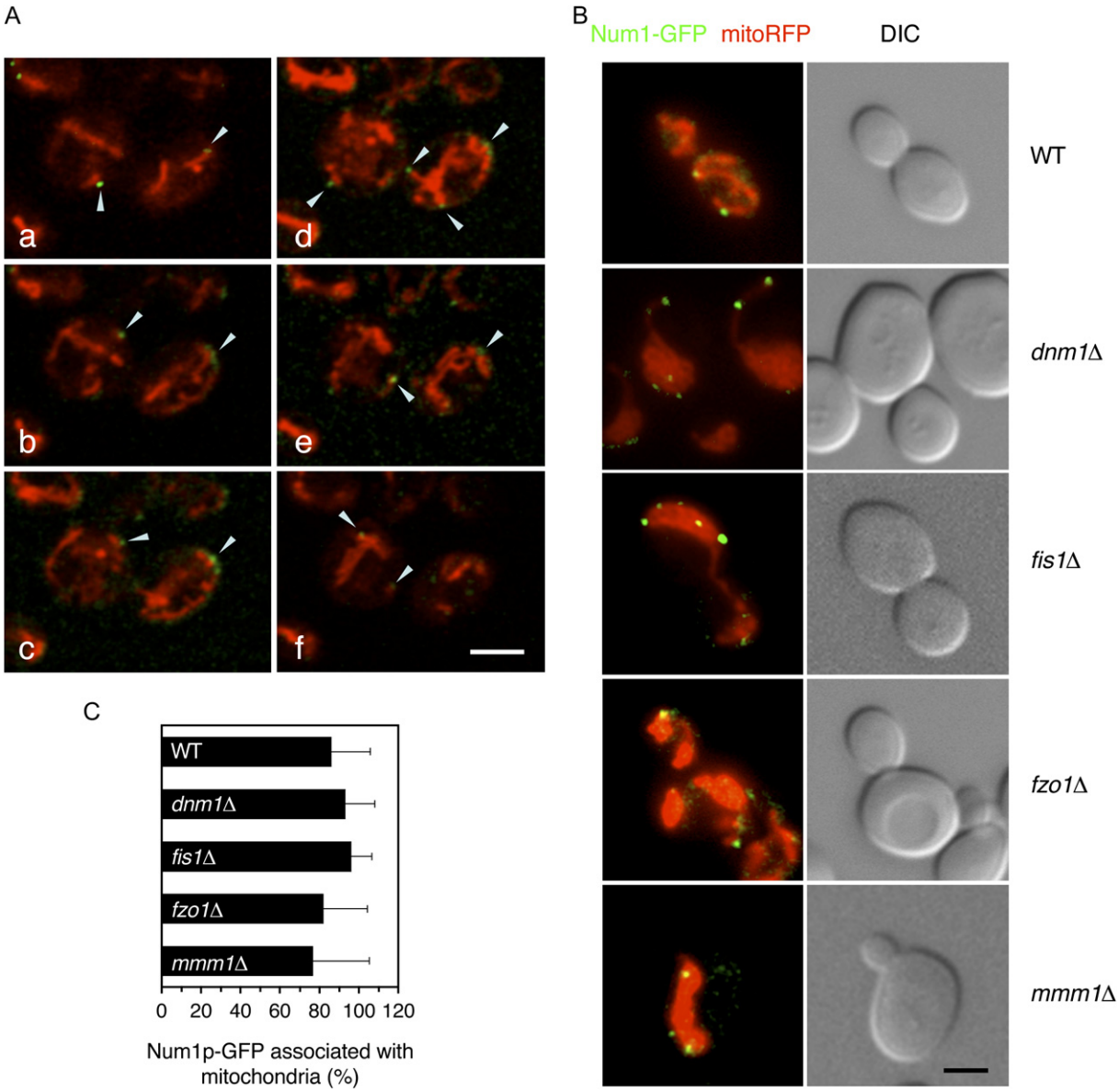


Figure 5. Num1p Is Associated with Mitochondria

(A) Confocal images at 0.6 μm intervals of Num1p-GFP strain RJ2131with pHS78 (Cox4p-RFP) are shown.

(B) Wild-type strain RJ2132, *dnm1Δ* strain RJ2146, *fis1*Δ strain RJ2248, *fzo1Δ* strain RJ2299, and *mmm1Δ* strain RJ2300, each expressing Num1p-GFP, were examined by wide-field fluorescence microscopy. Cells also expressed matrix-targeted RFP from pHS78 (WT, *dnm1Δ* and *fis1Δ* strains) or pHS300 (*fzo1Δ* and *mmm1Δ* strains). The scale bars are 3 μm.

(C) Quantitation of Num1-GFP puncta that clearly associated with mitochondria. At least 24 cells of each type were examined. Error bars indicate SD.

S4) were taken by fluorescence microscopy. The motion of Dnm1-GFP dots in a single wild-type or *num1Δ* cell was tracked for 1 min. In wild-type cells, we found that most of the Dnm1p-GFP particles were stationary or exhibited only small displacements, with only a few large Dnm1p-GFP dots moving abruptly. In *num1Δ* cells, we found that nearly all of the Dnm1p-GFP particles moved, and they moved more rapidly than those in wild-type cells (Figures 6C and 6D; compare Movies S1–S4). In addition, the Dnm1p-GFP particles appeared to move more randomly and traveled much shorter distances before changing direction. These Dnm1-GFP movements depend on actin and intracellular ATP (Figures 6C and 6D; Movies S2 and S5) (Jensen et al., 2000). We found that Lat A treatment slowed the motion of Dnm1p-GFP particles in *num1Δ* cells. These residual movements are not simply due to Brownian motion since elimination of cellular ATP by sodium azide treatment almost completely stopped the movement of Dnm1p-GFP (Movies S3 and S6). The effect on mitochondrial dynamics is not due to a general

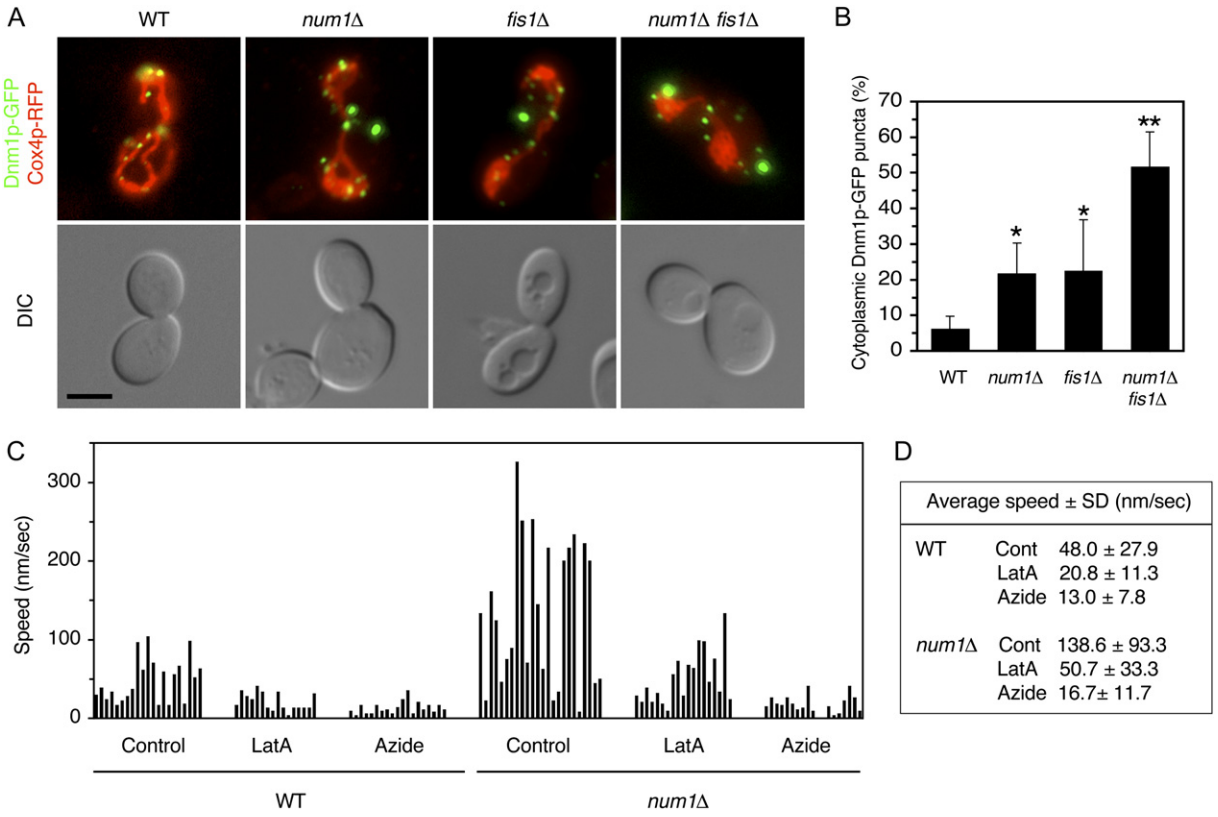


Figure 6. Num1p Is Required for Normal Dnm1p Distribution and Dynamics

(A) The association of Dnm1p with mitochondria requires Num1p and Fis1p. *dnm1Δ* strain YHS19, *num1Δ dnm1Δ* strain RJ2136, *fis1Δ dnm1Δ* strain RJ1566, and *num1Δ fis1Δ dnm1Δ* strain RJ2301, each expressing Dnm1p-GFP from pHS20, were stained with mitofluor red 589 and examined by fluorescence microscopy.

(B) Quantitation of the number of cytoplasmic Dnm1p-GFP particles in *NUM1*, *num1Δ*, *fis1Δ*, and *num1Δ fis1Δ* cells. At least 27 cells of each type were examined. For each cell, the number of Dnm1p-GFP puncta that clearly separate from mitochondria was divided by the total number of Dnm1p-GFP dots per cell; values shown are the mean ± SD. Single mutants were significantly different from wild-type cells (*$p < 0.0001$, Student's t test), and *fis1Δ num1Δ* double mutants were significantly different from either *fis1Δ* or *num1Δ* mutants (**$p < 0.0001$, Student's t test). Error bars indicate SD.

(C) Num1p controls the speed of Dnm1p movements. Time-lapse images of Dnm1p-GFP in wild-type and *num1Δ* cells were taken after 45 min treatments with DMSO (Control), Latrunculin A (Lat A), or sodium azide (Azide). Clearly visible Dnm1p-GFP spots were tracked for 60 s, and their average speeds were calculated. Each bar indicates a single Dnm1p-GFP particle. Full-length movies (Movies S1–S6) are available with this article online.

(D) The average speeds of all Dnm1p-GFP movements were calculated and are shown ± SD. At least 25 cells for each treatment were examined.

disruption of the cytoskeleton, since the actin distribution is normal in *num1*Δ cells (Figure S1). Given that our data show a fraction of Dnm1p-GFP moving in Lat A-treated cells, Num1p appears to also regulate the actin-independent motion of Dnm1p.

num1Δ dnm1Δ Mutants Are Defective in Cell Growth and Mitochondrial Inheritance

Previously, a genome-wide synthetic lethal screen reported that *num1Δ dnm1Δ* cells are inviable (Tong et al., 2004). However, in our strain backgrounds, we were able to construct the *num1Δ dnm1Δ* double mutant. We did find that these cells exhibit a temperature-sensitive growth defect, even on YEPD medium. At 23°C or 30°C, *num1*Δ, *dnm1*Δ, and *num1*Δ *dnm1*Δ cells grew at similar rates (not shown); however, at 35°C, the *num1*Δ *dnm1*Δ mutant grew slowly compared to wild-type, *num1*Δ, and *dnm1*Δ cells (Figure 7A). Strikingly, the growth defect was not seen when *num1*Δ was combined with mutations in other division components. The *num1*Δ *fis1*Δ and *num1*Δ *mdv1*Δ double mutants, as well as the *num1*Δ *mdv1*Δ *caf4*Δ triple mutant, grew normally at 35°C (Figure 7A). Our results therefore suggest that Dnm1p has an additional activity not shared with Fis1p, Mdv1p, or Caf4p.

In addition to growth defects, we also found that mitochondrial segregation is aberrant in *num1*Δ *dnm1*Δ cells. Cells were pregrown at 23°C and shifted to 35°C, and the distribution of their mitochondria was monitored by fluorescence microscopy (Figure 7B). After 4 hr at 35°C, ~20% of *num1Δ dnm1Δ* cells that completely lacked mitochondria were seen. Staining cells with calcofluor, which detects the bud scars on mother cells (Sloat et al., 1981), showed that mothers frequently lacked all mitochondria, with the organelles instead accumulating in the daughter buds (Figure 7C). In contrast to the double

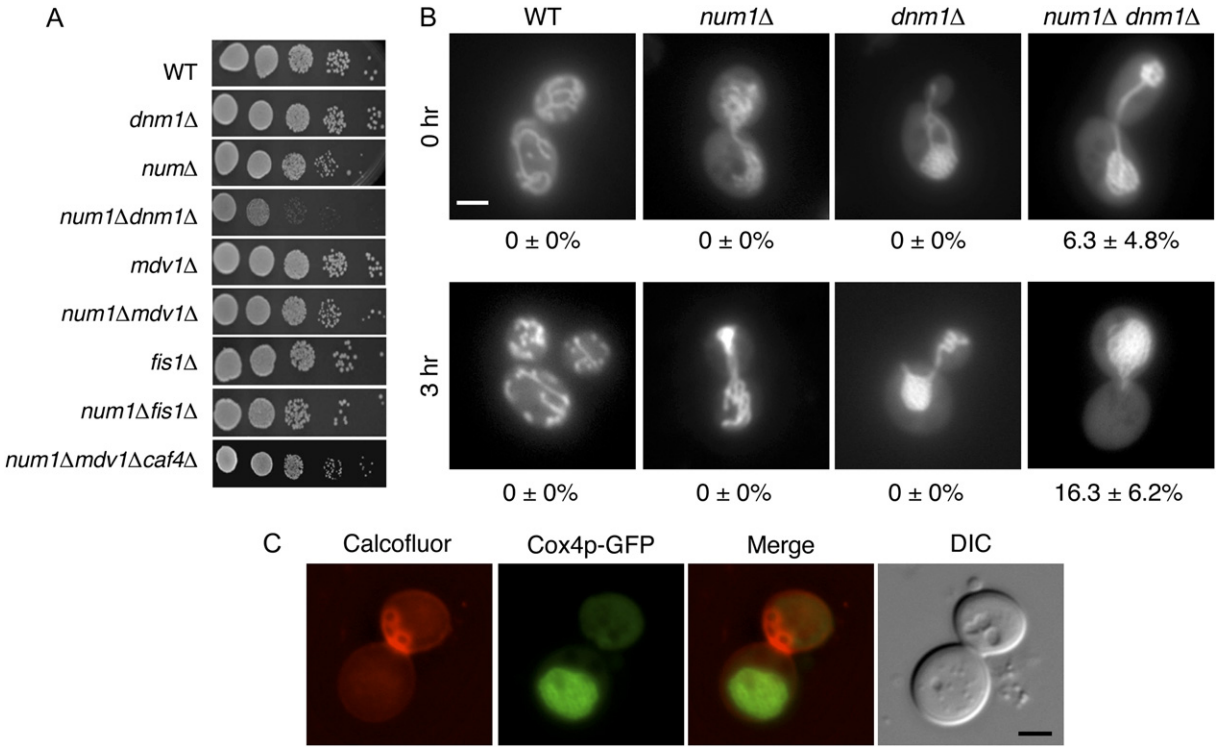


Figure 7. Mitochondrial Distribution Is Defective in *num1Δ dnm1Δ* Cells

(A) *num1Δ dnm1Δ* cells exhibit a severe growth defect at high temperature. Using a spot dilution assay, wild-type cells (RJ2137) and the single (*dnm1*Δ, RJ2135; *num1*Δ, RJ2134; *mdv1*Δ, RJ1253; *fis1*Δ, RJ1356), double (*num1*Δ *dnm1*Δ, RJ2136; *num1*Δ *mdv1*Δ, RJ2140; *num1*Δ *fis1*Δ, RJ2257), and triple mutants (*num1*Δ *mdv1*Δ *caf4*Δ, RJ2261) were plated on YEPD and incubated at 35°C for 3 days.

(B) *num1Δ dnm1Δ* cells exhibit defects in mitochondrial inheritance. Cells expressing Cox4p-GFP from pHS12 were grown at 24°C, shifted to 37°C for 3 hr, and examined by DIC and fluorescence microscopy. The numbers indicate the mean percentage and SD of cells lacking mitochondria ($n > 150$).

(C) *num1*Δ *dnm1*Δ cells fail to retain mitochondria in mother cells. Strain RJ2136 expressing Cox4p-GFP was grown at 24°C and then shifted to 37°C for 3.5 hr. Cells were stained with 0.1 mg/ml calcofluor for 5 min and were examined.

mutant, *dnm1Δ* and *num1Δ* single mutants showed no defects in mitochondrial inheritance at high temperatures, nor did cells in which *num1*Δ was combined with *fis1*Δ, *mdv1*Δ, or *caf4*Δ (not shown). Our results therefore show that faithful transmission of mitochondria during cytokinesis requires the action of Num1p and Dnm1p, but that it is not dependent on Mdv1p, Caf4p, or Fis1p.

DISCUSSION

To probe the role of the dynamin-related Dnm1 protein in mitochondrial division, we isolated high-copy suppressors of the dominant *DNM1-109* mutant. In addition to genes encoding the known division proteins Dnm1p and Mdv1p, we isolated Num1p, a protein previously shown to play a role in nuclear migration. Although we do not know the exact mechanism by which *NUM1* suppresses *DNM1-109*, we propose that overproduction of Num1p, Dnm1p, and Mdv1p negates the toxic effect of Dnm1-109 via direct binding of the individual proteins to Dnm1-109p, thus allowing productive interactions between the remaining division components. This idea is supported by the observations that Dnm1p self-assembles (Fukushima et al., 2001; Ingerman et al., 2005) and binds to Mdv1p (Cerveny et al., 2001; Tieu and Nunnari, 2000). Supporting the idea that Num1p and Dnm1p interact, we find that both proteins colocalize in the cell and copurify from cell extracts. We speculate that the Num1p-Dnm1p interaction is dynamic since only ~70% of Num1p associates with Dnm1p, since the Num1p- and Dnm1p-containing particles attach in a side-by-side manner, and since only a fraction of Dnm1p copurifies with Num1p.

Although Dnm1p clearly binds to mitochondria via Fis1p-Mdv1p-Caf4p complexes (Cerveny and Jensen, 2003; Cerveny et al., 2001; Griffin et al., 2005; Mozdy et al., 2000; Tieu et al., 2002), several studies have found that some amount of Dnm1p remains associated with mitochondria in the absence of Fis1p, Caf4, or Mdv1p. Our observations that the fraction of Dnm1p found in the cytosol increases in *num1*Δ mutants and that Num1p binds to mitochondria independent of Fis1p, Mdv1p, and Dnm1p suggest that Num1p provides an additional attachment site for Dnm1p. Moreover, since ~50% of Dnm1p-GFP remains bound to mitochondria in cells lacking both Num1p and Fis1p, additional anchors for Dnm1p await discovery. Since Num1p binds the plasma membrane via its C-terminal PH domain (Yu et al., 2004), our findings also provide an explanation for the earlier observations

that mitochondria reside at the cell cortex (Stevens, 1977), and the recent findings that most of the mitochondria-associated Dnm1 protein is at the cell periphery (Schauss et al., 2006).

Previous research has illustrated that Num1p functions as a cortical anchor in the daughter cell for the dynein motor, facilitating microtubule-dependent transport of the nucleus from the mother cell to the emerging bud (Bloom, 2001; Farkasovsky and Kuntzel, 2001; Heil-Chapdelaine et al., 2000). We find that *dyn1*Δ mutants have normal mitochondrial shape and distribution (K.L.C., unpublished data). Thus, Num1p's role in mitochondrial dynamics differs from its role in nuclear migration in that it does not require dynein. Instead, Num1p cooperates with Dnm1p in the mother cell to retain mitochondria. The partitioning of mitochondria during cell division requires actin-dependent migration from mother to daughter cell, as well as the attachment of a portion of the mitochondria to the cortex of both mother and daughter cells (Boldogh et al., 2001, 2004; Itoh et al., 2002; Yang et al., 1999). Our observations suggest that Num1p and Dnm1p are important for the retention of mitochondria in mother cells, since failure to make this attachment leads to the accumulation of mitochondria in the bud. Whether Num1p and Dnm1p also connect mitochondria to the daughter cell plasma membrane is unclear. Moreover, since *num1*Δ *dnm1*Δ cells are only partially defective in mitochondrial distribution, it is likely that other yeast proteins are involved. Among the possible candidates are two OM proteins, Mmm1p and Mdm10p, which also appear to help retain mitochondria in mother cells (Yang et al., 1999), as well as Myo2p, Mmr1p, and Ypt11p, which facilitate the bud-directed movement and daughter cell anchoring of mitochondria (Boldogh et al., 2004; Itoh et al., 2002, 2004).

In addition to organelle segregation, our observations indicate that Num1p also plays an important role in mitochondrial fission. First, the net-like mitochondria morphology in *num1*Δ cells is virtually identical to that in the *dnm1*Δ mutant. Second, like overproduction of Dnm1p or Mdv1p, high-copy *NUM1* plasmids suppress the mitochondrial division defect of the *DNM1-109* mutant. Finally, our time-lapse microscopy clearly shows that *num1Δ* mitochondria divide much less frequently than those in wild-type. Thus, although Dnm1p binds separately to Num1p and to the Mdv1p-Fis1p complex, mitochondrial scission and segregation appear somehow connected. For example, the cell cycle-specific regulation of Num1p expression (Farkasovsky and Kuntzel, 1995) raises the possibility that controlled mitochondrial fission at the bud site may help ensure that mitochondria remain in the mother cell after cytokinesis. We propose that a Num1p-mediated interaction with actin is required for mitochondrial segregation and division. Supporting this idea, Num1p is known to associate with Bni1, an actin-binding protein involved in cytoskeletal rearrangements (Bloom, 2001). Moreover, the interplay between dynamin and actin during the membrane scission events of endocytosis is well documented (Orth et al., 2002), and disruption of the actin cytoskeleton causes mitochondrial fragmentation mediated by the dynamin-related Dnm1 protein (Cerveny et al., 2001; Jensen et al., 2000). Our current results showing that *num1* mutants show an actin-dependent increase in Dnm1p movement also suggest that Num1p coordinates the interplay with the actin cytoskeleton. Together with our data, the recent discovery of Inp1p, a novel protein that participates in both peroxisomal inheritance and division (Fagarasanu et al., 2005), suggests that coupling between fission and transmission may be a common theme for the correct partitioning of a variety of organelles.

EXPERIMENTAL PROCEDURES

Strains and Relevant Genotypes

All strains and oligonucleotides used in this study are listed in Tables S1 and S2, respectively. The *MATa fzo1::kanMX4* strain, RJ2113, was constructed in W303-1Aby PCR-mediated gene disruption (Longtine et al., 1998) of *FZO1* with the *kanMX4* module that was amplified from pRS400 (Sikorski and Hieter, 1989). The *MATα DNM1 DNM1-109-HIS3* strain, RJ2116, was created by integrating XbaI-digested pKC90, which contains *DNM1-109-HA* on a *HIS3* plasmid, into W303-1B (Thomas and Rothstein, 1989) so that homologous recombination formed a tandem repeat of the *DNM1* and *DNM1-109-HA* in the chromosome. RJ2113 and RJ2116 were mated, the resulting diploids were sporulated, and *MATα* strain YKC3B and *MATa* strain YKC2C were generated by sporulation and dissection. To construct Num1p-GFP (strain RJ2132) and Num1p-RFP (strain RJ2193), cassettes encoding GFP or RFP, respectively, were PCR amplified from pSS10 (see below) or Y135 (see below) and inserted proximal to the stop codon of *NUM1* in strain FY833. *num1::kanMX4* strain RJ2131 was constructed by PCR-mediated gene disruption in FY833. The *fis1::HIS3* Num1p-GFP strain RJ2248 was constructed by PCR disruption of *FIS1* in strain RJ2132, while *dnm1::HIS3* strains, RJ2146 and RJ2194, expressing either Num1-GFP or Num1-RFP, respectively, were created by using PCR-mediated disruption of *DNM1* in strains RJ2132 and RJ2193. The *mmm1Δ* Num1p-GFP strain RJ2300 was created by crossing *dnm1*::*HIS3* Num1-GFP strain RJ2146 to *mmm1::URA3* strain 483 (Aiken Hobbs et al., 2001). *fzo1Δ* Num1p-GFP strain RJ2299 was created by inserting the GFP cassette from pSS10 immediately after the stop codon of *NUM1*. For double mutant analyses, *kanMX4* disruption strains purchased from Open Biosystems were mated, sporulated, and dissected. Disruption markers in tetratype tetrads were confirmed by PCR analysis generating strains RJ2134–RJ2145. *num1Δ mdv1Δ caf4Δ* strain RJ2261 was constructed by crossing the *caf4*Δ knockout strain (Open Biosystems) to RJ2140. *num1Δ dnm1Δ fis1Δ* strain RJ2301 was constructed by PCR disruption of *DNM1* in *num1Δ fis1Δ* strain RJ2257. Standard yeast genetic and media techniques were used (Adams et al., 1997).

Isolation of High-Copy Suppressors of *DNM1-109*

*ade2 ura3 fzo1*Δ *DNM1-109 DNM1* strain YKC3B was transformed with a library containing genomic DNA fragments in the 2μ-*URA3* plasmid pRS202 (Sikorski and Hieter, 1989). A total of 20,000 Ura^+ transformants were selected and screened for colony color. Since *ade2* strains accumulate a red pigment but *ade2* strains that lose mtDNA are white (Reaume and Tatum, 1949), 300 white Ura^+ colonies were patched to SD-ura media and replica plated to YEP glycerol/ethanol medium. Candidates that failed to grow on YEPgly/eth were collected. To control for random mtDNA loss, candidates were then mated to *ade2 ura3 fzo1*Δ *DNM1-109 DNM1* strain YKC2C. Plasmid-containing diploids that remained white were collected, and plasmid DNA was isolated. PCR and DNA sequence analysis showed that among the different plasmids we isolated were those expressing known division genes, *DNM1*and *MDV1*, as well as a plasmid (pKC999) containing an unexpected gene, *NUM1*.

DNA sequence analysis showed that the yeast DNA insert in pKC999 contained several different ORFs in addition to the incomplete *NUM1* gene. We constructed a 2μ-plasmid (pKC1000, see below) that carried only the truncated *NUM1* gene from pK999 (encoding the first 1380 amino acids of Num1p—called Num1Np). We found that pKC1000 induced rapid mtDNA loss in strain YKC3B, indicating that high levels of Num1Np are sufficient to suppress *DNM1-109*. Similarly, full-length *NUM1*, carried on 2μ-plasmid pKC1001 (see below), suppressed *DNM1-109*, as did 2μ-plasmids solely expressing *DNM1* (pKC1005 or pKC1008), *FIS1* (pKC1006), or *MDV1* (pKC1007).

YDR149c is a small ORF on the opposite DNA strand within *NUM1*, classified by the *Saccharomyces* Genome Database (http://www.yeastgenome.org) as a dubious ORF. Expression of the Ydr149c protein from either *CEN*-based or 2μ-plasmids did not restore normal mitochondrial morphology in *num1Δ* cells (K.L.C., unpublished data). Therefore, we conclude that the phenotypes seen in *num1Δ* cells are due to the lack of *NUM1*, but not *YDR149C*.

Plasmid Constructions

pKC90 was constructed by cloning a PvuI DNA fragment containing *DNM1-109-HA* from pHS69 (Jensen et al., 2000) into PvuI-cut pRS303 (Sikorski and Hieter, 1989). pKC1000, which expresses the first 1380 amino acids of the 313 kDa Num1 protein (Num1Np), was PCR amplified from genomic DNA (Hoffman and Winston, 1987) with oligonucleotides 1817 and 1818, and then inserted into XhoI/NotI-cut pRS426 (Sikorski and Hieter, 1989). pKC1001, which expresses full-length Num1p, was constructed with oligonucleotides 1916 and 1917 to amplify a DNA fragment encoding the C-terminal portion of Num1p. The resulting PCR product was cotransformed into yeast with NotI-digested pKC1000, and homologous recombination (Oldenburg et al., 1997) regenerated full-length *NUM1*.

pKC1002, which expresses *YDR149c* (but not *NUM1*), was constructed by amplifying a DNA fragment containing the predicted ORF as well as 500 bp upstream and 300 bp downstream with oligonucleotides 607 and 608, digesting the fragment with XhoI and NotI, and inserting it into a XhoI/NotI-cut pRS415 (Sikorski and Hieter, 1989).

2μ-*URA3-DNM1* (pKC1005) or 2μ-*LEU2-DNM* (pKC1008) was constructed by homologous recombination in yeast by first isolating *DNM1-HA* on a PvuII fragment from pHS14 (Sesaki and Jensen, 1999) and then cotransforming it into yeast with either XhoI/NotI-cut pRS426 or pRS425, respectively. pKC1006 was constructed by amplifying *FIS1* from yeast genomic DNA with oligonucleotides 650 and 675, digesting the PCR product with XhoI and BamHI, then inserting it into XhoI/BamHI-cut pRS425 (Sikorski and Hieter, 1989). pKC1007 was generated by amplification of *MDV1* with oligonucleotides 405 and 407, digestion with XhoI and NotI, and ligation into XhoI/NotI-cut pRS424 (Sikorski and Hieter, 1989).

Plasmid Y135 was created by substituting DsRed for the GFP cassette in pFA6a-GFP-kanMX6 (Longtine et al., 1998) as follows. DsRed was PCR amplified from pDsRed.T1.N1 (Bevis and Glick, 2002) with oligonucleotides 1077 and 1078, digested with Asc1/PacI, and inserted into AscI/PacI-cut pFA6a-GFP-kanMX6. After amplification of DsRed.T1 with oligos 461 and 462, pHS78 was made by inserting the PCR product into XbaI/NotI-cut pHS12 (Sesaki and Jensen, 1999). pSS10, which carries the red-shifted eGFP cassette, was constructed by PCR amplifying a 1056 bp PacI/BglII fragment from pSS5 (Youngman et al., 2004) with oligonucleotides 1518 and 1519 (Youngman et al., 2004), and it was used to replace GFP in PacI/BglII-cut pFA6a-GFP-TRP1 (Longtine et al., 1998). The ADH1-Su9-RFP plasmid pHS300 was constructed by inserting the XhoI/XbaI ADH1-Su9 cassette from pRS313-ADH1-Su9-GFP (Sesaki et al., 2006) and the XbaI/NotI RFP fragment from pRS78 (Youngman et al., 2004) into XhoI/NotI-digested pRS315 (Sikorski and Hieter, 1989).

pHS27 was constructed by amplifying *DNM1* from genomic DNA with oligos 268 and 269, followed by insertion into XhoI/NotI-cut pAA3. pHS21 was generated by cloning *DNM1-109*, amplified from genomic DNA with oligos 268 and 269, into XhoI/NotI-cut pAA1. Plasmids pHS20 (Sesaki and Jensen, 1999), pKC11 (Cerveny et al., 2001), pKC62 (Cerveny and Jensen, 2003), pAA1 (Aiken Hobbs et al., 2001), pAA3 (Aiken Hobbs et al., 2001), and Sec63p-GFP (Prinz et al., 2000) have been described.

TAP Tag Purification of Num1p

Yeast cells expressing the TAP tag (Rigaut et al., 1999) fused to the carboxyl terminus of Num1p (Open Biosystems) and control cells with no TAP tag were grown to late log phase and lysed in 50 ml buffer (1% digitonin [Calbiochem], 150 mM NaCl, 1 mM EDTA, 20 mM HEPES-KOH [pH 7.4], and protease inhibitor cocktail [Sigma]) by using an Avestin C-3 homogenizer according to manufacturer's instructions. Alternatively, cells were frozen in liquid nitrogen and then lysed in Triton X-100-containing buffer as described (Rigaut et al., 1999). Cleared lysate was incubated with IgG-Sepharose resin (Sigma) at 4°C with gentle rotation for 90 min, washed with lysis buffer, and eluted with SDS sample buffer. Lysate and eluate were analyzed by SDS-PAGE followed by western blotting with polyclonal antiserum directed toward Dnm1p (Cerveny and Jensen, 2003), Mdv1p (Cerveny and Jensen, 2003), OM45p (Jensen and Yaffe, 1988), and Fzo1p (Sesaki et al., 2003).

Microscopy

Yeast cells were observed during log phase growth in media containing 2% galactose or 2% raffinose. Wide-field fluorescence and differential interference contrast (DIC) images were captured with an Orca ER CCD camera (Hamamatsu) mounted on an Axioskop microscope with a 100× objective (Carl Zeiss, Inc.) and OpenLab software v3.0.8 (Improvision). For time-lapse images, cells were embedded in a thin layer of 2% low-melting temperature agarose (GIBCO-BRL) in SGal medium and covered with a glass coverslip. A total of 34 images were captured at regular intervals for 1 min with 2 × 2 binning. Confocal images were captured by using a Zeiss 510 confocal microscope with a 100× objective.

Mitochondria were visualized in cells by expressing matrix-targeted Cox4-GFP from pHS12 (Sesaki and Jensen, 1999) or matrix-targeted Cox4-RFP from pHS78 (Youngman et al., 2004). Alternatively, mitochondria were detected by treating cells with 10 nM mitofluor red 589 or 0.1 μg/ml $DiOC_6$ (both from Molecular Probes). Actin patches and cables were detected after fixing cells with 3.7% formaldehyde at room temperature for 90 min, washing with PBS, and staining with 1 μM Alexa 594 phalloidin (Molecular Probes).

To depolymerize the actin cytoskeleton, cells were treated with 250 μM Latrunculin A (Molecular Probes) for 45 min at room temperature. Staining cells with 1 μM Alexa phalloidin verified that actin cables and patches were disrupted.

Supplemental Data

Supplemental Data include time-lapse microscopy movies of Dnm1p-GFP as well as additional information for strain and plasmid construction, including oligonucleotide sequences, and are available at http://www.developmentalcell.com/cgi/content/full/12/3/363/DC1/.

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